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Commentary

Ectopic Calcification

Gathering Hard Facts about Soft Tissue Mineralization

Cecilia M. Giachelli

From the Pathology Department, University of Washington, Seattle, Washington

Ectopic calcification is defined as inappropriate biomineralization occurring in soft tissues.[1] Ectopic calcifications are typically composed of calcium phosphate salts, including hydroxyapatite, but can also consist of calcium oxalates and octacalcium phosphate as seen in kidney stones.[2] In uremic patients, a systemic mineral imbalance is associated with widespread ectopic calcification, referred to as metastatic calcification.[3] In the absence of a systemic mineral imbalance, ectopic calcification is typically termed dystrophic calcification. Often, these sites show evidence of tissue alteration and/or necrosis. Dystrophic mineralization is commonly observed in soft tissues as a result of injury, disease, and aging. Although most soft tissues can undergo calcification, skin, kidney, tendons, and cardiovascular tissues appear particularly prone to developing this pathology.[4] In addition, a number of prosthetic devices are prone to ectopic calcification, as discussed below. Recent insights into the mechanisms regulating ectopic calcification have come from studies of cardiovascular calcification, including that by Kim et al[5] in this issue of the *Journal*, and thus will be the major focus of this article. The reader is referred to other reviews for information about additional tissue-specific ectopic calcifications.[2,6,7]

Ectopic calcification can lead to clinical symptoms when it occurs in cardiovascular tissues, particularly arteries and heart valves. In arteries, calcification is correlated with atherosclerotic plaque burden and increased risk of myocardial infarction,[8–10] increased ischemic episodes in peripheral vascular disease,[11] and increased risk of dissection following angioplasty.[12] Medial arterial calcification is also a strong independent marker of future cardiovascular events in diabetic patients.[13] In the heart, valves are particularly prone to calcification. Degenerative calcific aortic stenosis is currently the most common valvular lesion encountered in clinical cardiology and one of the most difficult to manage.[14] It is estimated that approximately 1–2% of the elderly population suffer from this pathology, which is characterized by encrustation of aortic valve leaflets with apatitic mineral deposits and subsequent stiffening, tearing, and mechanical failure. Congenital anomalies, inflammatory changes such as those seen in rheumatic fever, renal disease, and age are all risk factors for aortic valve stenosis.[14]

The definitive treatment for severe symptomatic aortic stenosis is aortic valve replacement. This treatment was put into clinical practice in the 1960s and has resulted in dramatic improvement in longevity and symptoms of patients with valve disease, but problems persist. More than 40,000 patients undergo valve replacement each year in the United States.[15] Two types of prosthetic valves are commonly used: mechanical valves and tissue bioprosthetic valves. Mechanical valves are typically made of materials such as pure titanium, cobalt-chromium alloys, and pyrolytic carbon. These implants offer excellent long-term durability but are procoagulant and prothrombotic, thus necessitating chronic anticoagulation therapy and limiting their use in many patients (eg, women of childbearing age and children). The major bioprosthetic tissue valves used clinically include valves fabricated from chemically cross-linked animal tissues, such as porcine aortic valves. In addition, non-cross-linked human aortic valve allografts are used, but usually in limited supply. Although tissue bioprosthetic valves have superior hemodynamic and thromboresistant properties compared to mechanical valves, those fabricated from porcine aortic valves or bovine pericardium have a higher rate of failure. Failure is most often attributed, again, to calcification of the tissue prosthetic valve. In fact, by 10 years, one third of bioprosthetic valves require replacement, increasing to two thirds by 15 years. In addition, failure in children often occurs within 2 to 5 years, and is increased substantially in hemodialysis patients.[3,15–18]

The need for improvements in tissue bioprosthetic valves aimed at minimizing valve failure and patient reoperation rates has driven research in this area and provided much of the current information on mechanisms of ectopic calcification. These studies have led to an

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Address reprint requests to Cecilia M. Giachelli, Ph.D., Associate Professor, Pathology Department, University of Washington, Seattle, WA 98195. E-mail: ceci@u.washington.edu.

excellent understanding of the morphology, ultrastructure, and crystal chemistry of bioprosthetic valve mineralization. Morphologically, nearly all explanted bioprosthetic valves display loss of cuspal connective tissue cells and endothelium, valve calcification particularly in the spongiosa and at the commissures, and very little inflammation.[19–22] Using transmission electron microscopy, several studies have shown that explanted porcine xenografts contain intracytoplasmic and interstitial calcospherulae, calcified collagen fibrils, and platelike calcium deposits on amorphous material.[23,24] The calcified deposits were identified by X-ray diffraction as apatitic in nature,[24] similar but not identical to those observed in natural heart valve calcification.[25] In addition, a number of animal models have been developed to study bioprosthetic valve calcification, including subcutaneous implantation in rats[26,27] and rabbits,[28] as well as valve replacement in sheep or calves.[27] Using these models, biological determinants of cardiac valve calcification have been identified and include host factors (eg, young age, uremia, hyperparathyroidism), tissue fixation conditions (eg, aldehyde cross-linking), and mechanical stress.[15,29] These animal models have also been used to test various anticalcification therapies.[29] Despite these considerable achievements, lack of knowledge of the underlying mechanisms controlling abnormal biomineralizations has hampered the development of clinically effective anticalcification strategies.

Though mammalian extracellular fluids are metastable with respect to hydroxyapatite, this crystal does not spontaneously precipitate. Thus, bone and tooth mineral, as well as ectopic calcifications, exist in disequilibrium with the blood.[30] This paradox has led to the concept of heterogeneous nucleation, whereby biomolecules (termed "nucleators") may serve as substrates for initial crystal formation.[31] In bone and teeth, nucleators are thought to be generated by active processes in osteogenic cells as part of their normal physiological function. However, bioprosthetic implants are devoid of live cells. Thus, a key question is how bioprosthetic valve calcification is initiated, ie, what are the nucleators?

Important observations have led to several mechanistic theories. First, aldehyde fixation appears to be a prerequisite for bioprosthetic valve mineralization. Animal studies have shown that nonfixed or alternatively processed (eg, by photo-oxidation) valves do not mineralize following subcutaneous implantation, and processed but non-glutaraldehyde-fixed human allografts show much less mineralization than aldehyde-fixed valves.[26,32–34] Therefore, it has been proposed that aldehyde cross-linking may create specific nucleation sites in valve extracellular matrix that are highly susceptible to nucleation. Furthermore, residual glutaraldehyde may leach out of fixed valves and induce surrounding tissue injury, thus promoting mineralization (glutaraldehyde toxicity).[35–37] Second, devitalization of aldehyde-treated prosthetic valves has been proposed to alter membrane permeability and calcium influx,[38,39] thus bringing high concentrations of calcium into contact with high phosphate levels in membrane-bound intracellular compartments and achieving $[Ca^{+2}] \times [PO_4^{-3}]$ products high enough for precipitation. Finally, mechanical stress is proposed to exacerbate mineralization, because regions of valves with the greatest stresses correlate with the highest degree of mineralization.[23,24] However, studies showing that bioprosthetic valves mineralize in noncirculatory *in vivo* models indicate that mechanical stress is not required for calcification to occur.[26–28]

Conversely, several potential mechanisms have been ruled out by the experimental data. While some recent studies on ectopic mineralization suggest that host mesenchymal cells may contribute to mineralization (see below), this is apparently not required in bioprosthetic valve mineralization because fixed valve leaflets implanted within millipore diffusion chambers go on to mineralize.[26,40] Likewise, a role for nonspecific inflammation or specific immunity as causes of mineralization appears unlikely, because both fixed and nonfixed tissue valves show a foreign body response consisting of predominantly mononuclear infiltrates following implantation, yet only fixed valves mineralize.[41] In addition, valve tissues implanted in congenitally athymic, T cell-deficient (nude) mice calcify to the same extent as implants in immunocompetent mice.[42] Thus, graft rejection is not thought to play a major role in bioprosthetic valve mineralization.

The study by Kim and colleagues[5] in this issue of the *Journal* provides hard data supporting the calcium influx theory and simultaneously provides a provocative idea about the role of aldehyde fixation in bioprosthetic valve mineralization. Porcine aortic valve fibroblasts were isolated and glutaraldehyde-fixed. Using calcium-sensitive dyes, an immediate and sustained increase in cytosolic Ca^{2+} was measured in fixed cells compared to live cells. In 0.6% glutaraldehyde-treated valve fibroblasts, intracellular concentrations of calcium reached ~1.5 mmol/L, a million times greater than calcium levels seen in unfixed fibroblasts. Furthermore, a severalfold increase in Pi was also noted within the fixed valve cells, bringing the Pi concentrations to ~35 mmol/L, and greatly elevating the $[Ca^{+2}] \times [PO_4^{-3}]$ product. With time, the glutaradehyde-fixed cells progressively depleted the media of Ca^{2+} and inorganic phosphate, and mineralization of the cultures was observed. The phenomenon relied on the presence extracellular Ca^{2+} and was shown to depend on the concentration of glutaraldehyde. Thus, these are the first studies to experimentally measure increased intracellular Ca^{2+} and inorganic phosphate levels following glutaraldehyde fixation, and to correlate these changes to valve cell mineralization, thus substantiating the calcium influx theory.

The study by Kim et al[5] additionally provides insights into a possible mechanism of glutaradehyde-induced mineralization. The investigators noted cellular blebbing following glutaraldehyde fixation, and an increase in calcein fluorescence intensity in these membranous structures increased as these structures calcified. Ultrastructural analyses found that calcium crystals were contained in the blebs, either in close association with the inner surface of the plasma membrane or in swollen mitochondria. With time, the entire valve fibroblast cell was observed to contain mineral deposits. Thus, the authors propose that following glutaraldehyde-induced calcium

influx, cell blebbing is induced and serves to isolate the overloaded calcium, and that it is in these structures that nucleation of apatite first occurs. Temporal studies in experimental bioprosthetic valve calcification are consistent with this possibility because calcification is observed first in devitalized cells and cell fragments, and only later in matrix collagen fibers.[27] Importantly, ultrastructural analysis of failed porcine xenografts provides clinical evidence for the initial calcification in cellular debris and membrane fragments of porcine cusp cells.[24] Furthermore, physiological mineralization of bone and cartilage is thought to proceed, at least in part, via nucleation in matrix vesicles and cell derived membranous vesicles, either actively released by live cells or resulting from apoptosis.[43] Mineralization of matrix vesicles and cell degeneration products have also been observed in diseased aortic valves in addition to calcified atherosclerotic plaques.[44] Whether the membrane blebs observed in the studies by Kim et al[5] are analogous to matrix vesicles either in derivation or function, however, is not yet known. Likewise, whether glutaraldehyde might induce apoptotic cell death is unclear. Finally, it should be stressed that although nucleation in cell-derived membranes following glutaraldehyde fixation appears to be the most important mechanism early in bioprosthetic valve mineralization, it is impossible to rule out a potential contribution of cross-linked valve tissue matrix to nucleation of apatite, especially late in bioprosthetic valve calcification.

Despite the overwhelming *in vitro* and *in vivo* data supporting the calcium influx theory of bioprosthetic valve calcification, this hypothesis does not predict the delayed onset of calcification-related problems observed in human patients and experimental models of bioprosthetic valve calcification. This may be explained, in part, by the fact that glutaraldehyde fixation of heterografts is usually performed in the absence of Ca^{2+}, perhaps limiting bleb formation and subsequent mineralization. However, it is also likely that natural inhibitors of calcification are present in the host, and that it is only when these inhibitory mechanisms are overcome that calcium crystals precipitate and proliferate.

The idea that natural inhibitors of mineralization exist has long been recognized by investigators in the hard tissue field,[4,45,46] but only recently have definitive data for systemic or local inhibitors of cardiovascular calcification been obtained. Using gene knockout technology, mice null for the matrix gla protein (MGP) gene were created and found to have extensive cardiovascular calcification, in addition to abnormal cartilage calcification. In fact, MGP-null mice die within the first 2 months of age due to arterial rupture and heart failure as a result of extensive calcification of the large elastic and muscular arteries and heart valves.[47] In wild-type mice, MGP is normally expressed at high levels in cartilage and blood vessels.[48] Likewise, osteoprotegerin-null mice were recently shown to develop arterial calcification in addition to osteoporosis.[49] Osteoprotegerin is a soluble member of the TGF receptor super family, and known to regulate osteoclast differentiation. In addition to expression in bone, osteoprotegerin is also normally expressed in blood vessels.[49] Other genes whose mutation leads to enhanced cardiovascular calcification have been reported and include glucosidase,[50] desmin,[51] and carbonic anhydrase II.[52] The mechanisms by which loss of these genes leads to enhanced susceptibility of cardiovascular calcification is not yet known, but represent important areas for further research. Finally, osteopontin, an acidic phosphoprotein found at high levels in calcified vascular tissues (see below) was recently shown to be a potent inhibitor of mineralization of bovine aortic smooth muscle cells *in vitro.*[53] Osteopontin is not normally expressed in blood vessels but is rapidly induced on injury.[54] Thus, these data suggest that MGP, osteoprotegerin, and osteopontin may be naturally occurring inhibitors of cardiovascular calcification which are either constitutively expressed (surveillance inhibitors) or induced (damage control inhibitors) in arteries to prevent ectopic mineralization.

As mentioned above, ectopic calcifications are often associated with cell death; however, not all ectopic calcifications occur in obviously devitalized tissues, including those observed in native aortic valve stenosis[55] or in the Monckeberg's type calcification seen in blood vessels from diabetic and uremic patients.[56,57] In addition, in both advanced aortic valve and atherosclerotic lesions, outright bone formation, though rare, has been noted.[58,59] Furthermore, bone matrix and morphogenic proteins thought to be involved in regulating normal osteogenesis have been described in calcified vascular tissues, including osteopontin,[54,55,60–63] osteonectin,[62] matrix gla protein,[47,48] osteocalcin,[26,64] bone sialoprotein,[65] and bone morphogenic protein 2a.[66] Finally, vascular cells that can undergo mineralization *in vitro* have been isolated and shown to resemble osteoblasts in that they express several genes thought to be important for bone mineralization.[66–69] These observations have led investigators to revise the concept that vascular calcification is a purely degenerative disease and have suggested that specific mechanisms regulating soft tissue calcification exist.[70–72]

In summary, ectopic calcification is a common problem associated with organ injury, disease, and bioprosthetic implants, with or without degenerative changes evident within the tissue. In all cases, cells appear to play an active role in regulating mineral deposition. Cells may regulate nucleation by synthesizing a mineralization-competent matrix, by actively releasing matrix vesicles, or by dying and providing cellular degeneration products (as in the case of bioprosthetic valve cells), thereby stimulating crystal nucleation. Conversely, cells appear to synthesize natural inhibitors of mineralization that may normally serve to prevent ectopic mineralization. It is no doubt the balance between these pro- and anti-calcification mechanisms that dictates the formation of ectopic calcification at a given site. Gaining a better understanding of these mechanisms should lead to improved prevention and treatment of ectopic calcification in the future.

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